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THE USE OF THE URN CELL COMPLEXES OF *SIPUNCULUS NUDUS* FOR THE DETECTION OF THE PRESENCE OF MUCUS STIMULATING SUBSTANCES IN THE SERUM OF RABBITS WITH MUCOID ENTERITIS*

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Abstract

Urn cell complexes (UCC) from the marine coelomate *Sipunculus nudus* were used to measure a progressive change in mucus stimulating substances (MSS) in the serum of rabbits with mucoid enteritis. The serum, but not plasma, of rabbits with mucoid enteritis induced significant hypersecretion in UCC. Neither diluted nor undiluted serum or plasma from control animals induced hypersecretion. The length of the mucus tail induced in the urn cells by serum of affected rabbits was directly related to the severity of the disease. When the MSS was partially purified by cold precipitation and gel filtration, its molecular weight was determined to be between 10,000 and 13,500 daltons. These studies establish the urn cell assay as a system in which abnormal secretion of mucus can be monitored and followed with non-invasive *in vitro* techniques.

Introduction

Although aberrations in the secretion of mucus play important roles in many disease processes of animals and humans, the mechanisms that regulate the secretion of mucus in different physiological and pathological states is poorly understood. Factors found in body fluids such as serum have been implicated in the pathogenesis of several diseases in which mucus hypersecretion is a feature (Franklin and Bang, 1980; Kurlandsky *et al.*, 1980; Bang *et al.*, 1983). Serum factors were first suggested to be a possible stimulus to mucus secretion by Johnson (1935) who showed that a measurable increase in the volume of tracheal secretions could be obtained by running a film of blood over the mucous membrane of cat trachea. Hall *et al.* (1978) demonstrated that this increased tracheal secretion came from both submucosal glands and from the goblet cells, the two sources of mucus secretion in the trachea. They suggested that since serum exudates are often found in sputum or bronchial washings of patients suffering a wide range of respiratory diseases, it is likely that there are stimulants of augumented mucus secretion in the serum of these patients.

Bang and Bang (1979) have developed an *in vitro* assay system using the urn cell complexes (UCC) of the marine coelomate, *Sipunculus nudus,* to search for factors which regulate mucus secretion including factors found in serum in different

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pathological states. Urn cell complexes, complexes of ciliated and mucus secreting cells, are a normal component of the coelomic fluid of *S. nudus*. Cantacuzéne (1922) observed that bacterial infections of *S. nudus* induced its UCC to hypersecrete mucus. Using the UCC assay, mucus stimulating substances (MSS) have been demonstrated in normal human serum heated to 85°C for 4 minutes (Bang and Bang, 1971), in human lacrimal fluids (Franklin and Bang, 1980) and in other body fluids (Bang and Bang, 1979). Titers of MSS in serum (Kurlandsky *et al.*, 1980; Bang *et al.*, 1983) and lacrimal fluids (Franklin and Bang, 1980) have also been found to be altered in certain disease states.

The present study was designed (1) to determine if there are MSS in the blood of rabbits with mucoid enteritis (ME), a naturally occurring disease of rabbits in which mucus plugs of the small intestine is pathognomonic (Fig. 1); (2) to determine if these factors have a relationship with the disease; and (3) to isolate, purify, and partially characterize any such factors.

Materials and Methods

Buffer

PBS (phosphate buffered saline: 0.010 *M* sodium phosphate, 0.15 *M* sodium chloride, pH 7.4) was prepared fresh in deionized water with monobasic and dibasic sodium phosphate and sodium chloride (Biological grade, Fisher Scientific, Silver Spring, MD).

FIGURE 1. Colon of rabbit with mucoid enteritis showing mucus impaction.

Animals

The collection and maintenance of *S. nudus* have been previously described (Bang and Bang, 1972). In brief, *Sipunculus nudus* were dug from the sand at the lowest monthly ebb tide in their natural habitat at Loquemeau, Finistere, Station Biologique, Roscoff, France. They were maintained in France in large tanks of running sea water with beds of sand from their native area. *S. nudus* were flown to the United States packed in thermos bottles and were maintained in Instant Ocean (Aquaria Aquarium Systems, Inc., Eastlake, Ohio) in artificial sea water. They have been maintained in this fashion for up to one year while still yielding viable urn cells. New Zealand white (NZW) rabbits from our colony, with clinically diagnosed mucoid enteritis, and normal aged matched NZW rabbits were used in these studies.

Blood collection

Rabbits were bled from the marginal ear vein and the blood was placed in either 15 ml or 50 ml polypropylene centrifuge tubes (Falcon Plastic, Oxnard, CA). The blood was allowed to clot at 4°C for 4 hours. The blood was then centrifuged at $200 \times g$ for twenty minutes at room temperature and the serum removed and placed into 15 ml polypropylene tubes, and stored at −70°C. Blood was also heparinized and plasma collected in a similar fashion. In one experiment, plasma was also obtained by placing blood into 3 ml glass tubes containing EDTA (4 mg) (BD, Rutherford, NJ), sodium heparin (BD), or potassium oxalate (6.0 μg potassium oxalate with 7.5 mg sodium fluoride) (BD).

Urn cell complexes

Stocks of UCC for *in vitro* tests were obtained by withdrawing 3 ml of coelomic fluid from *S. nudus* with a 20 gauge needle and putting aliquots into polypropylene 400 μl microcentrifuge tubes (Falcon Plastic). When the heavy blood cells settled, the clear supernatant contained thousands of freely swimming urn cells. There were 6–20 urns/μl of clear supernatant. The preparations remained viable for 3–4 weeks when stored at 4°C.

Urn cell complex assay

Urn cell complexes were used in an assay to detect MSS in the serum and plasma of rabbits. Serum and plasma were usually diluted 1:40 in boiled filtered sea water (BFSW) to make them isotonic with *S. nudus* coelomic fluid and were used unheated. In titration experiments, serum and plasma were used either undiluted or in serial two-fold dilutions. In testing for MSS, 20 μl of the test fluid were added to 20 μl of urn fluid in a well of a glass depression slide. The length of the mucus tail induced in the urn cell by a given time was measured by an eyepiece micrometer in a light microscope and was expressed in multiples of the average diameter of the vesicle cell. Twenty to 30 UCC were counted and a mean tail length was determined. All test substances were coded and all tests were conducted without knowledge as to the source of the test material. A known MSS, heated human serum diluted 1:40 in sea water, was used as a positive control. Boiled filtered sea water (BFSW) and unheated human serum were used as negative controls. All tests were read 15 minutes after the stimulus was added.

Isolation of MSS

Blood was collected from rabbits with mucoid enteritis and from control rabbits as described above and placed into polypropylene test tubes. The blood was allowed to clot and serum was removed and placed in a polypropylene test tube which was subjected to a high speed centrifugation (91,000 × *g*) at 4°C for 3 hours yielding a pellet which contained the mucus stimulating activity. This pellet was resuspended in 10 ml of PBS (pH 7.4) and allowed to stand at 4°C overnight. A slow speed spin (200 × *g*) at 4°C for 20 min yielded the mucus stimulating activity in the pellet. This pellet was resuspended at 7.5 ml of PBS and was gel filtered on Sephacryl S-200 Superfine (Lot No. 4119, Pharmacia Fine Chemicals, Uppsala, Sweden). The glass column (Pharmacia Fine Chemicals, Uppsala, Sweden) was siliconized (Prosil-28, PCR Research Chemical Inc.). The column (1.6 × 92.5 cm) was equilibrated with PBS (pH 7.4) at room temperature. The flow rate of the column was 16 ml/h and 2.3 ml fractions were collected in polypropylene tubes using a LKB 7000 Fraction collector and a duostaltic pump (Buchler Instruments, Fort Lee, NJ). The column was standardized with beef liver catalase (MW 240,000, Boehringer-Mannheim, New York, NY), transferrin (MW 82,000, Sigma Chemical Co., St. Louis, MO), egg albumin (MW 45,000, Boehringer-Mannheim, New York, NY), soybean trypsin inhibitor (MW 21,500, Worthington Biochemical, Freehold, NJ), cytochrome C (MW 12,500, Boehringer-Mannheim, New York, NY), and K_2CrO_4 (MW 194.2, Scientific Products, Columbia, MD). Each collected fraction was tested for absorbance at 206, 220 and 280 nm, on a Beckman model 25 spectrophotometer. Fractions were also tested in the urn cell assay.

Dialysis of MSS

Samples of collected fractions were transferred to cellulose dialysis tubing with an exclusion limit of 1000 daltons (43 mm × 10 mm in 1% NaBenzoate, Spectrum Medical Industries, Inc., Los Angeles, CA). The sample was dialyzed against 1000 ml of 1 mM NH_4HCO_3 at 4°C for 36 hours with two solution changes, one every 12 hours. The dialysis solution was stirred continuously throughout the process. Samples were rapidly frozen in a dry ice/acetone bath and then lyophilized.

Protein determination

Column fractions were subjected to protein determination by the Bio-Rad Protein Assay. Gammaglobulin (Bio-Rad Laboratories) was used as a standard.

Trypsin treatment of MSS

Column fractions containing MSS activity were subjected to a 0.1% trypsin (Sigma Chemical Co., St. Louis, MO) solution (final concentration) for 15 minutes at room temperature. At the end of 15 minutes, Soybean Trypsin Inhibitor (SBTI) (Worthington Biochem Corp., Freehold, NJ) was added to the mixture, 1 mg SBTI for each 1.53 mg of trypsin, and allowed to stand for 15 minutes at room temperature. The resulting mixture was then tested in the UCC assay as described above. Trypsin and SBTI were also allowed to react in PBS and the resulting complex was mixed with equal volumes of fractions containing MSS activity and was used in the UCC assay. The effect of 0.1% trypsin on preformed UCC tails was also examined.

Stability of MSS

Fractions from the Sephacryl S-200 column were heated to 85°C for 10 minutes to test their heat stability. Sephacryl S-200 fractions with MSS activity were also subjected to three rapid freeze/thaw cycles.

Results

Urn cell response to serum or plasma collected during the natural outbreak of mucoid enteritis

Blood was collected and prepared as described above. Serum, but not heparinized plasma, from rabbits with mucoid enteritis induced significant hypersecretion in the urn cells (Fig. 2). Undiluted and diluted serum or plasma from control animals failed to induce mucus tails when used in the UCC assay (Table I). Control serum heated to 85°C for 5 minutes also failed to induce mucus tails. The length of the mucus tails induced by the serum of the affected animals correlated well with the clinical grading of the disease (Table II).

Plasma and serum

To address the question of whether the absence of MSS in the plasma of rabbits with mucoid enteritis was due to an artifact of using heparin as a anticoagulant, plasma was prepared from the blood of rabbits with mucoid enteritis using sodium citrate and EDTA, as well as sodium heparin. Sera were also recovered from these animals. Plasma from rabbits with MSS activity in their serum was negative for MSS activity regardless of the anticoagulant used. The plasma, undiluted or diluted 1:20 or 1:40 in BFSW was also heated to 85°C for 5 minutes without generation of MSS activity.

Partial characterization of MSS

Sephacryl S-200 fractionation. Sera from 10 ME positive rabbits with MSS activity were pooled and again tested for UCC activity. The pooled sera induced mucus tails that were three times the diameter of the vesicle cell (graded +3). The pooled sera was centrifuged as previously described. MSS activity was found only in the resuspended pellet. The resuspended pellet was then subjected to gel filtration on Sephacryl S-200 at room temperature. The sera of control animals were treated in the same manner (Fig. 3). Upon gel filtration, the resuspended cold precipitate from experimental sera yielded two minor peaks, each with a small amount of absorbance at 280 nm and molecular weights of 97,000 and 25,000. Both peaks, however, lacked MSS activity in the UCC assay. Mucus stimulating activity was detected in the MW range of 11,000–13,000. All active fractions lacked absorbance at 206, 220, or 280 nm. The percentage of MSS activity placed on the column and recovered was 82.0%. All fractions of control sera lacked MSS activity.

When the entire procedure was repeated twice with sera from additional animals with ME, MSS activity was found in fractions having a MW range of 10,750 to 13,500 and 10,000 to 11,000. The percentages MSS recovered in the experiments were only 42.8% and 47.5%, respectively. Control sera continued to yield no MSS activity in any fraction.

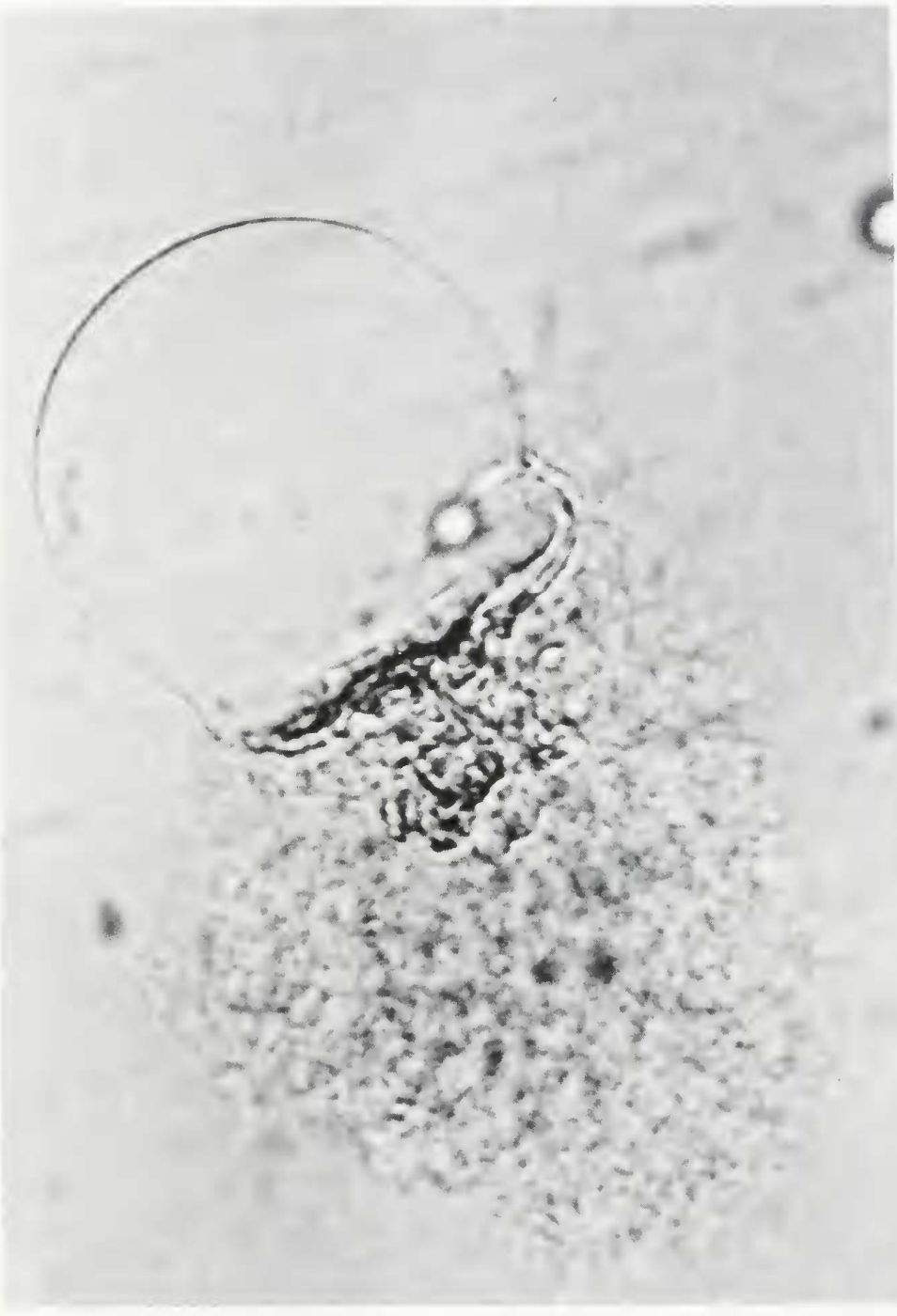

FIGURE 2. Cell complex of *Sipunculus nudus* stimulated to hypersecrete mucus by incubation with serum of rabbits with mucoid enteritis. The serum was diluted 1/40 in BFSW and mixed with an equal volume of coelomic fluid containing approximately 10 UCC/ml. After 10 minutes of incubation, 20 to 30 UCC's were counted and a mean tail length was determined.

TABLE I

Urn cell response to blood from rabbits with mucoid enteritis

Group	Serum	Plasma
Controls[a]	0/30	0/30
Mucoid enteritis[b]	13/13	0/13

[a] Controls included 20 apparently healthy rabbits, 5 rabbits with enteric disease as evidenced by diarrhea but not mucoid enteritis, and 5 rabbits with *Pasteurella* infections of the upper respiratory tract.

[b] Rabbits were clinically diagnosed as having mucoid enteritis. The diagnosis was based on the passage of copious amounts of mucus, anorexia, and polydipsia, symptoms diagnostic for the disease.

Physical properties of MSS

Aliquot of Sephacryl S-200 fractions containing MSS activity (MW 12,000–13,000) were combined, dialyzed, lyophilized, and reconstituted in PBS with no loss of MSS activity. Aliquots of MW 11,000 which also contained MSS activity were heated at 37°C, 56°C, or 85°C for 10 minutes with little or no change in MSS activity. Sera heated in a similar fashion also maintained its MSS activity. Sera and purified MSS heated to 100°C for 10 minutes, however, lost activity. The purified MSS and MSS containing sera withstood three rapid freeze/thaw cycles with no loss of activity. Purified MSS remained stable for at least 7 days at −70°C and for 3 days at 4°C. Attempts to reprecipitate purified MSS by standing in PBS at 4°C for 12 hours failed. However, when inactive MW 97,000 fraction was mixed with purified MSS (MW 12,000–13,000) and allowed to stand at 4°C for 12 hours, a

TABLE II

Urn cell response and clinical grading of mucoid enteritis

Rabbit number	Urn cell[a] response	Clinical[b] grading
320	+	1[c]
326	+	1
342	+	1
325	++	2
322	++	3
324	++	3
336	++	3
340	++	3
315	+++	4
337	+++	4
338	+++	4
341	+++	4
345	+++	4

[a] Response to a 1:40 dilution of sera from rabbits with mucoid enteritis.

[b] Rabbits passing small amounts of mucus and showing no other clinical signs were graded 1. Rabbits passing small amounts of mucus as well as showing signs of anorexia and polydipsia were graded 2. Rabbits passing copious amounts of mucus and showing signs of polydipsia and anorexia were graded 3. Rabbits which were no longer passing mucus after 2–3 days of passing copious amounts and whose abdomens were distended with impacted intestines were graded 4.

[c] The length of the mucus tail induced in the urn cell was measured by an eyepiece micrometer and was expressed in multiples of the average diameter of the vesicle cell (1 = 50 μm).

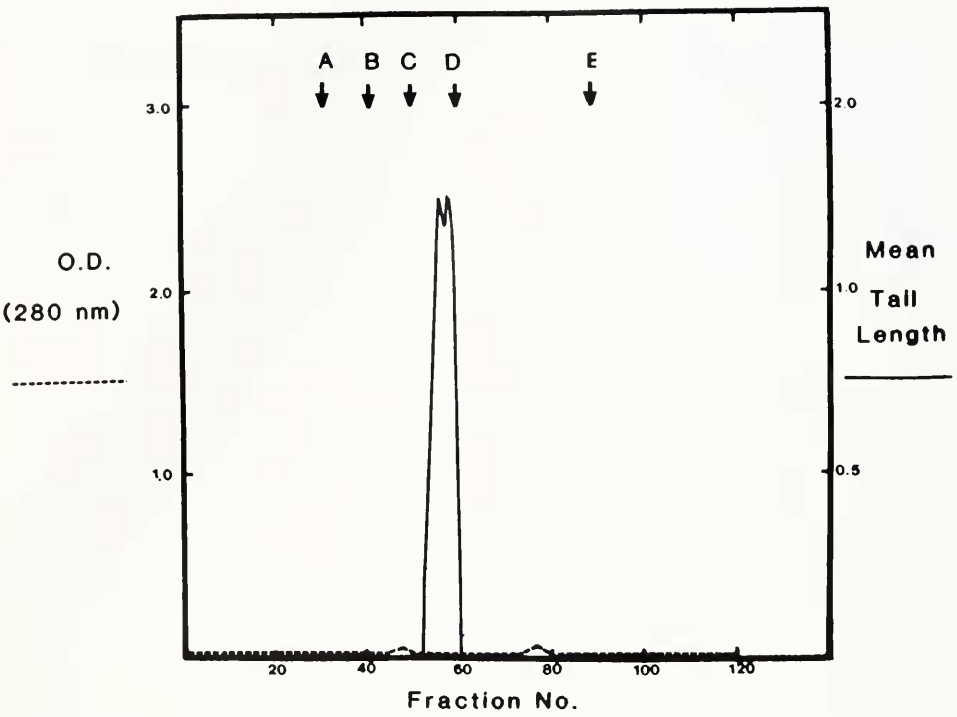

FIGURE 3. Blood was collected from rabbits with mucoid enteritis and allowed to clot. Serum was collected and subjected to a centrifugation at 91,000 × *g* at 4°C for 3 hours. The pellet was resuspended and allowed to stand at 4°C overnight. The cold precipitate formed at 280 nm and presence of MSS activity was determined for each fraction. A = beef liver catalase (MW 240,000); B = transferrin (MW 82,000); C = egg albumin (MW 45,000); D = soybean trypsin inhibitor (MW 21,500); E = K_2CrO_4 (MW 194.2).

cold precipitate was formed with MSS activity found in the resuspended pellet following centrifugation. Purified MSS activity could be neutralized by treatment with 0.1% trypsin for 30 minutes at room temperature. When trypsin and SBTI were allowed to complex and then added to the MSS, there was no inhibition of MSS activity. Preformed mucus tails were not cleaved by 0.1% trypsin.

Bio-Rad assay for protein in the Sephacryl S-200 fractions from rabbits with mucoid enteritis

The Bio-Rad assay was used as a method of determination of the concentration of protein in the fractions of sera from rabbits with mucoid enteritis. Small amounts of protein (2.0–3.2 ng/μl) were present in fractions containing MSS activity. Similar amounts (2.0–4.0 ng/μl) of these proteins, however, were found in comparable fractions of serum from control rabbits.

DISCUSSION

These studies of MSS in sera of rabbits with mucoid enteritis represent the first attempt to study the progressive changes in MSS titer during the course of an acute

disease which causes excess mucus secretion in experimental animals. The very close correlation between MSS in the serum and the disease as measured by (1) the 100% presence of the factor in the serum of animals with mucoid enteritis and the absence in all controls and (2) the correlation of severity of the disease and the intensity of the UCC response makes it extremely likely that the UCC does, indeed, measure a substance of significance in the pathogenesis of this disease characterized by excessive intestinal mucus secretion.

MSS purified from the sera of rabbits with mucoid enteritis has a low molecular weight (10,000–13,500). The purest rabbit MSS active material obtained by ultracentrifugation, cold precipitation at 4°C, and gel filtration on Sephacryl S-200 yield low concentrations of protein as determined by absorbance at 280 nm and by the Bio-Rad assay for proteins (2–3 $\mu g/\mu l$). The apparent ability to inhibit MSS activity with trypsin, however, suggests that MSS is in fact a protein.

The MSS appears to be very stable. Heating either the whole active serum or active Sephacryl S-200 fractions at 85°C for 10 minutes did not affect the MSS activity, although heating MSS containing sera or purified MSS at 100°C for 10 minutes did abrogate the MSS activity. The MSS active sera and purified MSS were frozen and thawed three times without loss of activity. MSS fractions from the Sephacryl S-200 column could also be stored at 4°C for at least 72 hours without loss of activity. Purified MSS was dialyzed, lyophilized, and reconstituted with little apparent loss in activity.

The finding that MSS appears in the serum and not the plasma of animals with mucoid enteritis suggests that some change occurs during the clotting process, such as secretion from platelets, that is essential in the generation of MSS. Attempts to prepare plasma with a variety of anticoagulants failed to produce MSS in plasma of rabbits with mucoid enteritis. Attempts to isolate MSS from disrupted platelet suspensions of both diseased and control rabbits have been unsuccessful (results not shown). A possible role for platelets in the production or release of MSS remains, however, since platelet aggregation may be necessary for the release of MSS. Aggregation is accomplished experimentally by adding a substrate to the platelet suspension. This was not done in these studies and should be attempted in future studies. The interaction of platelets and the other cells of the clot also may be important in the generation of MSS. Alternatively, MSS may be generated by leukocytes during the clotting process. Finally, the interaction of the clotting process and the production of kinins as well as sequences in the complement cascade may be important in the generation of MSS.

Mucus stimulating substances have been demonstrated in heated normal human serum (Bang and Bang, 1980), unheated human tears (Franklin and Bang, 1980), and unheated filtrates of human cholera stools (Bang and Bang, 1979). It has also been found in unheated sea water dilutions of *Lotus tetragonolbus* lectin (Nicosia, 1979) and in unheated suspensions of sonicated human lymphoblastoid cells (Kulemann-Kloene *et al.*, 1982). Alterations in MSS profiles have been demonstrated in cystic fibrosis patients who demonstrate abnormal mucus secretion (Kurlandsky *et al.*, 1980; Bang *et al.*, 1983). The present studies suggest the use of the urn cell assay as a system in which such abnormal secretion of mucus can be monitored and followed with relatively non-invasive *in vitro* techniques.

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